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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

CAPILLARY BLOOD-PRESSURE AND THE GLOMERULAR FILTRATION THEORY.

LEONARD HILL AND JAMES McQUEEN,

National Institute for Medical Research, London.

Received for publication June 17th, 1921.

ACCORDING to Cushny,(1) "The function of the kidney may be shortly defined as the filtration of the non-colloid constituents through the capsule and the absorption of 'Locke's fluid' through the tubule cells. The capsule furnishes the tubules with the fluid as it exists in the circulation, the tubules return to the blood the fluid best adapted for the tissues, and allow the rest to escape in the urine."

The modern theory of the secretion of the urine, as enunciated by this author, stands or falls by an accurate measurement of the blood-pressure in the capillaries of the glomeruli. The theory postulates that protein-free plasma is filtered through each glomerulus without expenditure of energy by the cells which encapsulate it, the energy of filtration being derived from the action of the heart. Reabsorption of this fluid occurs through the vital activity of the renal tubule cells.

According to the modern theory, which assumes that the glomerulus is comparable to a gelatine filter, the possibility of filtration through it depends on (*a*) the actual capillary pressure available to separate the water, sugar and salts from the blood; (*b*) the osmotic pressure of the blood colloids, which may be put at about 25–30 mm. Hg., and which will be less according as the blood colloid is diluted.

Now, there are two well-established facts in the physiology of the kidney with which this filtration theory of the glomerular function must come into line. To take the first, the maximum to which the secretory pressure rises in the ureter and pelvis of the kidney lies between 60–70 mm. Hg. It may be lower; under conditions of saline diuresis it may be higher. But taking the figure of 60 mm. Hg. pressure, the modern view of kidney function recognises

that the capillary pressure in the glomerulus must, when the ureter pressure is 60 mm. Hg., reach a pressure of 90 mm. Hg., because the capillary pressure must over-top the ureter pressure by 30 mm. Hg., if the osmotic pull of the proteins on the water of the blood is to be overcome and filtration ensue. Accordingly the modern view, while admitting that this estimate of the glomerular capillary pressure is at the best mere guesswork, states that the capillary pressure may be taken as 20 per cent. below carotid pressure.

The assumption of a pressure 20 per cent. less than the aortic or carotid pressure is necessitated by a consideration of the second well-established fact in the physiology of the kidney. The minimum pressure in the aorta or carotid below which urine fails to be secreted from normal undiluted blood is about 40 mm. Hg. Deducting from the 40 mm. Hg. 20 per cent. of its value, a glomerular capillary pressure is left of 32 mm. Hg.—an amount capable of overcoming the osmotic pull of the blood colloids on the water and maintaining filtration.

Now let us consider what this assumption involves. If the carotid blood-pressure is 120 mm. Hg. the glomerular capillary pressure is 20 per cent. less, or 96 mm. Hg. If the aortic blood-pressure is 40 mm. Hg. the glomerular capillary blood-pressure is 20 per cent. less, or 32 mm. Hg. The loss of pressure in both cases is due to friction of the blood passing from arteries through arterioles to capillaries. The pressure gradient indicates the loss due to friction. Now, while the kidney is actively secreting it swells owing to vaso-dilation; even when the blood-pressure is very low, viz. 40 mm. Hg., the renal arterioles may be, and most probably are in a state of vaso-dilation. While the pressure gradient in the first instance is assumed to be 24 mm. Hg., in the second instance a pressure gradient of 8 mm. Hg. is postulated. It does not seem likely that the pressure gradient in the same vessels, and over the same course, should vary in this manner.

The consideration of these figures shows that the 20 per cent. reduction in aortic pressure is a figure arbitrarily chosen to suit the filtration theory of glomerular function. If the gradient were in both cases 24 mm. Hg., when the aortic or carotid blood-pressure is 40 mm. Hg., there would remain only 16 mm. Hg. available to separate blood colloid from the water, and filtration could not occur at that pressure as long as the colloidal pull reached its normal figure of 20–30 mm. Hg. The value of the gradient has to be sacrificed to maintain consistency in fundamental portions of the filtration theory.

In regard to the problem of the pressure values in the arteries, arterioles and capillaries of the mammalian kidney, only indirect proof is at present available as a basis of argument. But there are certain measurements that can be and have been accurately obtained. The pressure in the renal veins of a dog was found by Burton Opitz [2] to be 10·9 mm. Hg. We know from the work of L. Hill [3] on animals that a very low pressure pertains in the capillaries not only of the frog, but in the ear and abdominal muscular wall of the mouse and in the wing of the bat, and Hill and McQueen [4] have measured the arteriole pressure in the skin of the hand and this is roughly 10 mm. Hg. at heart level—one-third to one-fifth of almost all previous estimates. Basler [5] found the pressure of blood, flowing from a cut in the finger, raised the mercury in a manometer 7–9 mm. In cutting the finger arterioles will be

wounded, and this may be taken then to be the arteriole pressure. The pressure in the veins leading from the capillary areas of the hand in man is 0·7–6·7 mm. Hg., [6] that is a few millimetres below the arteriole pressure—sufficient to ensure a pressure gradient. Consequently it is reasonable to suppose that, with a renal vein pressure of 10·9 mm. Hg. the pressure in the second capillary network of the kidney lies in the neighbourhood of 12–13 mm. Hg.

It is possible to arrive at a fairly accurate estimate of the pressure in the small arteries of the kidney substance, for if the ureter is obstructed, we know that up to 40 mm. Hg. pressure in the ureter (no less pressure must pertain in the renal tubules and capsules) no interference with the blood-flow from the renal vein occurs (Cushny). At 50 mm. Hg. pressure Lindman found the venous outflow was impaired. Confined as the kidney is by its capsule, all the smaller arteries, including the afferent arteries of the glomeruli, the capillaries and venules, are compressed by this 50 mm. Hg. pressure which pertains from the pelvis through the tubules, less such part, if any, of the pressure as the *membranæ propriæ* of the tubules and capsules shield the vessels from. These membranes in the kidney do not withhold pressure to the same extent as in the salivary gland, where the secretory pressure can rise above aortic systolic pressure without arresting the circulation through the salivary gland. Consequently the blood-pressure in the venules and capillaries rises towards this 50 mm. Hg. pressure until the smaller arteries are diminished in calibre, and this occurs when the diastolic pressure in these arteries is overcome. Hence diminution of venous outflow means the overcoming of the diastolic pressure in the small arteries. We constructed a scheme in which this diminution of venous outflow was demonstrable on overcoming the diastolic pressure in the artery.[7] Now we know that the range between diastolic pressure and systolic pressure is limited in small arteries. Hence the systolic pressure in the small arteries of the kidney is probably not higher that 60 mm. Hg.

A pressure range of 50–60 mm. Hg. in the small arteries of the kidney is not very different from the pressure in the small arteries of the hand, or in the main artery of the proximal segment of the bat's wing, which has been directly measured by one of us, hence we are able to estimate the probable fall of pressure between these arteries and the capillary field of the glomerulus.

It has been generally held that the pressure of the two capillary fields athwart the blood path in the kidney requires of necessity a high blood-pressure to maintain circulation. Barcroft and Straub have shown that with large injections of saline a secretion of urine has taken place with no more than 12 mm. Hg. pressure in the aorta. Of course in this case the colloid of the blood was diluted and viscosity diminished, but it would appear that the long path of the double capillary field can be traversed by fluid at very low pressure. In this case the 20 per cent. reduction would reduce the glomerular capillary pressure to 10 mm. Hg. When the excised kidney is perfused at 15–20 mm. Hg. pressure, fluid escapes from the ureter in some quantity, and the perfusion fluid circulates through the two capillary fields. All this is in accordance with the findings of L. Hill, viz. that a pressure of 1–2 mm. Hg. suffices to make a flow in the capillaries of the web of the amputated leg of the frog. The theory

of great resistance in the capillary bed is erroneous. The pressure gradient does not fall much in capillary fields, but during the passage of blood through the smaller arteries and arterioles. L. Hill has found in the bat's wing that the pressure in arterioles branching at a right angle (the afferent artery to each glomerulus is such) falls to about one-half of the pressure in the main stems, *e.g.* from 50 to 20–25 mm. Hg. In the case of a schema with a narrow tube opening into a wider one and this in its turn into a narrow one, the lateral pressure in the wider part actually exceeds that in the entrance tube. Such a schema cannot be justly used to interpret the pressures in the vessels of the glomerulus, because the arteriole of the glomerulus divides into a large number of capillaries and these reunite into the afferent vessel, so that, as can be demonstrated in a schema so constructed, lower pressures exist in each individual capillary than pertain in the arteriole. Accordingly all the available data tend to show that if the small artery pressure be 50–60 mm. Hg., the pressure in the capillary field of the glomerulus falls as in other capillary fields which are accessible to direct measurement. Consequently the capillary field in the glomerulus has a blood-pressure at most some few millimetres higher than in the second capillary field. We have seen that, according to the modern theory of renal secretion, a pressure of 96 mm. Hg. is assumed for the glomerular capillaries when the aortic pressure is 120 mm. Hg. and we have reached the conclusion that the second capillary field in the kidney has a pressure a few millimetres above the renal vein pressure. It would be truly remarkable if, glomerular pressure being assumed by the theory to be 20 per cent. less than aortic, 24 mm. Hg. pressure were lost in passing from the aorta to the capillaries of the glomerulus, and 85 mm. Hg. pressure were lost in passing from the glomerulus through the second capillary field to the renal vein.

We now turn to another point. The modern theory supposes that a glomerular capillary pressure of some 90 mm. Hg. filters protein-free plasma through Bowman's capsule till the pressure in the obstructed ureter reaches 60 mm. Hg., when the filtration ceases, because some 30 mm. Hg. pressure is required to separate the water from the colloids of the blood. No attention appears to have been paid by those upholding this theory to the general condition of the kidney in this experiment. Cushny says that the pressure in the ureter is propagated along the tubules to the capsule, and this opposes the filtration pressure. It must equally be propagated through the walls of the tubules to the surrounding capillaries and venules. It will be found that the kidney becomes tense and hard, which is proof that every structure in it is under pressure, being confined within the resisting structure of the kidney capsule. Supposing the pressure in the glomerular capillaries rises to the maximum pressure in the ureter, viz. 60 mm. Hg., this can only result from the compression of venous exits by the pressure of the urine in the renal tubules and capsules. The high capillary pressure so induced is artificial, and does not indicate the normal conditions.

We may here recall an experiment of Henderson (5). He found the pressure in the ureter fell on inhibiting the heart, and ascribed this to retraction of the glomeruli and fluid in the tubules escaping into the capsules so enlarged. Against this view must be put the valvular action of the

openings of the collecting tubules into the pelvis, which, as Cushny has shown, prevent any escape of fluid from the pelvis even when the kidney substance is cut away almost till the pelvis is reached. The true explanation is that the pelvis becomes distended when the ureter is obstructed, and presses in upon the kidney, which resists compression owing to the high vascular pressure pertaining in arteries, capillaries and veins confined by the renal capsule. When the heart is inhibited the kidney's resistance to deformation is lessened, the pelvis swells, its capacity increasing, and so the pressure within it falls, as has been observed by J. McQueen and A. Webster.

We may also recall the interesting experiment of Alcock and Lewi of enclosing the kidney in plaster-of-Paris so that it cannot expand. Injection of saline caused diuresis, and the renal venous blood became brighter in colour. The conditions here are comparable to the brain or eye or salivary gland enclosed in a capsule strictly limiting expansion of the organ. Dilatation of arteries brings about constriction of veins, and the whole system—arteries, capillaries and veins—approximates to a rigid system with rapid blood-flow, which in its turn favours diuresis.

The important point to note is that when compression is applied over the whole organ the blood-flow is diminished so soon as the diastolic pressure in the small arteries is overcome. The compressed arteries then open intermittently with each beat of the pulse, and blood flows through them only so long as the level of systolic pressure overtops the compressing pressure, as has been observed in the frog's web, bat's wing, etc., by L. Hill. For this reason secretion ceases before systolic pressure can be attained in the ureter.

The pressure gradient deduced in the kidney when actively secreting and in a state of vaso-dilatation, according to the above argument, may then be stated thus:

Small arteries	50–60 mm. Hg.
Glomerular capillaries . .	15–20 „
Second relay of capillaries .	13–15 „
Renal vein	11 „

There remains for discussion certain corroborative evidence of the accuracy of these findings.

We are fortunate in having for consideration well-authenticated results due to various workers drawn from experiments on the action of saline injections into animals. Any consideration of the influence of the action of saline injections in inducing dilution of the blood colloids and promoting diuresis must carry with it the consideration of the influence of colloid dilution by salines on the capillaries of the body generally. When saline solutions are injected into animals, water and salts pass not only through the glomerular capillaries but through those of the body generally (Magnus). Saline injections permeate equally well through capillary systems where the capillary blood-pressure has been shown to be less than 10 mm. Hg. Dilution of the colloids of the blood has then a precisely similar effect where the capillary pressure is known to be low as it has in the case of the renal capillaries, *e. g.* of the glomerulus, wherein the blood-pressure, according to the modern view, is believed to be 20 per cent. below the aortic pressure. The sole difference in the two cases is that the water and the salts escape through the

ureter in the one case, and escape through the lymph into the blood-stream or are retained in the tissues in the other case. Evidently the physiological permeation of salts or water through capillaries can take place with the very low capillary pressure which has been determined by L. Hill. In place of filtration it might be argued that the water and salts pass through the capillary in virtue of the blood having a lower osmotic pressure than the tissue cell. With normal blood a colloidal osmotic pull of 20–30 mm. Hg. is assumed inside the capillary, but outside the capillary there stand the tissue cells—an osmotic counterbalancing pull. Dilute the colloid of the blood with saline, and the water and salts might be taken up by the cells by osmosis from a low capillary pressure. Knowlton claims that if 5 per cent. of gelatine in Ringer solution is injected with an osmotic pressure of 23 mm. Hg.—a figure pretty nearly the osmotic pressure of blood colloid—there is no diuresis; but on the modern theory the only means in such case of stopping an increased flow of urine would be to raise the osmotic pressure of the blood to 20 per cent. less than the arterial pressure, viz. 90 mm. Hg.!

It may be said that increased filtration through the glomerular capillaries does in fact take place, but the water and salts are what Cushny calls an optimal fluid, and so are absorbed in the tubules. Well, if such be the case, the modern view of kidney function expressly points out that the call for oxygen or increase of oxygen consumption follows on the absorption process, not the filtration process. Now it is pointed out by Knowlton that whether Ringer solution alone or Ringer solution *plus* 5 per cent. gelatine are injected the consumption of oxygen in each case is practically the same. In this experiment the modern view of glomerular function breaks down.

We may here note that Tamura and Miwa (9) find that physiological permeation of the kidney occurs without any increase of oxygen use.

To sum up, then, the diastolic pressure of small arteries in the mammalian kidney lies between 40–50 mm. Hg. The blood-pressure in the second capillary system lies close to the pressure observed by Burton Opitz in the renal vein (11 mm. Hg.). The capillary pressure in the glomeruli must lie between 40–50 mm. Hg. and 13–14 mm. Hg., and as a large drop of pressure exists elsewhere in the body between the small arteries and the capillary field, there is good reason to think that glomerular capillary pressure does not exceed 15–20 mm. Hg. The maximum secretory pressure depends on the compression exerted by the secretion in the tubules on the veins, capillaries and arteries of the kidney, and is determined by the diastolic pressure of the small arteries. When these arteries are compressed during diastole less blood gets through, venous outflow is diminished and the kidney cells cease to secrete.

The production of diuresis by saline injection and the failure of such by injection of saline *plus* 5 per cent. gelatine are against the filtration theory of production of urine, and suggest that diuresis depends on a physiological permeation. In the structure of the kidney there is no evidence of structure which would support the blood capillaries and allow them to act as a filtering membrane. Swelling of the tubules by compressing the venules will raise the pressure in the capillaries and venules up towards the arteriole pressure and cause a swift flow of blood through a more rigid tense system of vessels, the flow not being impeded until the diastolic arteriole pressure is overtopped.

The glomerular capsules and tubules are not empty spaces into which filtration can take place, but contain fluid. The pulse transmitted through the arteries expands the whole kidney confined within its capsule, and expresses this fluid towards the pelvis just as it impels venous blood out of the venules into the veins. Hence the relation of flow of urine to pulse pressure found by Erlanger and Hooker ([10, 11]) and by Gesell ([12]). The kidney enclosed by its capsule is comparable in this respect to the brain enclosed by the dura mater and cranial wall. We know that the blood pulses out of the cerebral venous sinuses and that the cerebro-spinal fluid, when connected with a gauge, shows similar pulses.

We now turn to experimental observations made by one of us (L.H.), who has observed the glomeruli of the frog's kidney under the microscope, watched the circulation and measured the glomerular capillary pressure.

Method.—A male frog is put under urethane, or has its skull crushed between the blades of artery forceps so as to destroy the brain, leaving the spinal bulb intact. The frog is stretched out on its side and a crucial incision made through the skin over the ilium on that side which is uppermost. The body-wall is cut through along the upper two-thirds of the ilium and this part of the bone removed. Any hæmorrhage is stopped by a cautery heated to a dull heat. The membrane at the outer edge of the kidney is then picked up in the forceps at a point about one-third above its lower end, and a ligature tied on there. The animal is then laid belly upwards on an apparatus similar to that designed by Graham Brown for the research which he carried out with Roy on capillary pressure.([13]) The apparatus consists of a small cylinder of brass with a glass floor, and a dome-shaped piece of transparent peritoneal membrane tied on "atop." Into the cylinder there enters a tube which is connected by a ⊥-piece with (1) a manometer, (2) a tube for blowing air in and raising the pressure. The brass cylinder forms the centre of a flat piece of metal which is covered with a sheet of cork, to which cork the frog's head and knee-joints are pinned suitably. The outer border of the kidney, by means of the thread attached to it, is gently drawn on to the peritoneal membrane which covers the top of the brass cylinder. The kidney is then wetted with Ringer's solution, and a flat piece of thin glass, attached to a holder (forming a part of the apparatus) is lowered down so as to cover the kidney. The kidney can now be compressed between the peritoneal membrane and the glass cover when air is blown into the cylinder. The apparatus is brought on to the stage of the microscope, and the kidney examined with the low power.

The blood will be seen streaming from the renal portal vein in copious slow streams over the surface from the outer to the inner border. Between these vessels glomeruli may be seen, sometimes very clearly on the outer border. On compressing the kidney by 2–3 mm. Hg. the venous blood is expelled and the glomeruli come more clearly into view. The blood corpuscles can be seen swirling through the glomerular network; the capsules and the afferent and efferent vessels may be visible in some cases. The arterioles supplying the glomeruli may also be observed crossing the kidney in successful preparations. A pressure of 5–10 mm. Hg. suffices to slow distinctly the flow in the glomeruli, while it takes 20–35 mm. Hg. to stop the flow in the arterioles supplying them. The glomerular capillary pressure is about as high as that found in the

papillæ of the frog's tongue, and only a little higher than that in the frog's web. It may be concluded, then, that the frictional resistance to the normal rate of flow in the glomerular capillaries is very small, just as it is found to be in other capillaries.

After injecting subcutaneously 1 c.c. of Ringer's solution, or normal saline, an hour before preparing the kidney for examination, the capsules are found distended with clear fluid equal to or even double the volume of the glomerulus. As it still takes a pressure of some 6 mm. Hg. to retard the glomerular circulation, it follows that the fluid distending the capsule is at a less pressure than this, and that it takes very little pressure to distend the capsule, which must be an easily distended structure. It follows, too, from these observations that the glomeruli are in truth the seat of physiological permeation or secretion of watery fluid, as has been generally surmised on other grounds, and that the fluid may come through much quicker than it passes away down the tubules. The latter do not appear distended with fluid. It is worth noting that the distended capsules are surrounded with capillary networks in which flows the blood from the renal portal veins. It seems possible that some interchange may go on between the intra-capsular fluid and the venous blood.

We may here note that Hamburger ([14]) claims that the glomerular permeability is physiological and selective. Perfusing the frog's renal arteries with Ringer solution, he finds a retention of glucose (added to the Ringer in the normal concentration pertaining to the blood) depending on the composition of the Ringer solution. If potassium ions are left out, for example, the glomerular epithelium becomes permeable to glucose. Another radio-active element can replace potassium just as Zwaardemaker claims to be the case with the frog's heart. While the glomerular epithelium retains glucose, it is permeated by levulose and other sugars.

CONCLUSIONS.

The paper deals with the modern theory of the secretion of urine, and evidence is brought forward to show that pressure in the glomerular capillaries is a low one, and that physiological permeation—not filtration—controls the passage of fluid through the glomeruli. Observations on the circulation in the frog's glomeruli are detailed and support these conclusions, which are also borne out by experiments of Hamburger, which demonstrate the selective action of the epithelium covering the glomeruli.

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THE THEORY OF AGGLUTININ ESTIMATION.

J. O. WAKELIN BARRATT, M.D., D.Sc.

From the Lister Institute, London.

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In the present communication the mathematical expression of (1) the action of agglutinin upon antigen, and (2) the decay of antigen, is considered in relation to the estimation of agglutinin.

THE AGGLUTININ-ANTIGEN REACTION.

I. The reaction between agglutinin and its antigen (bacteria, red cells, blood-platelets, etc.) was observed (Arrhenius (1905), on the basis of experiments at 37° C. by Madsen) to be in fair agreement with the equation—

$$yt = m \quad . \quad . \quad . \quad \text{(I)}$$

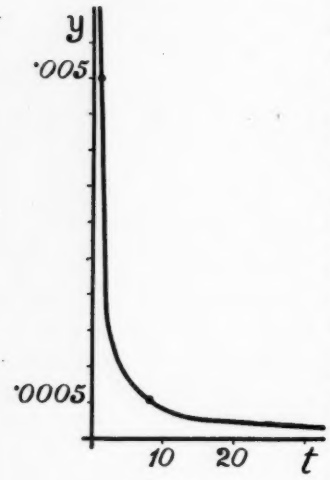

Fig. 1.

y representing the concentration of the agglutinin-holding serum employed, t the time of appearance of agglutination, and m being a constant. The curve given by this equation is a rectangular hyperbola (Fig. 1). The reaction recalls that of hydrochloric acid upon a marble surface: so long as the area of the surface remains unaltered and the amount of acid present is not appreciably changed equation (I) is applicable, the time required for a fixed amount of marble to dissolve being inversely proportional to the concentration of acid.

McKendrick (1911), in the course of a study of amboceptor action, reviewed the experiments quoted by Arrhenius, and advanced the view that the reaction between agglutinin and its antigen in reality proceeds at a bi-molecular reaction rate, represented by the equation—

$$yt = \frac{c^2 z}{cz - y} \quad . \quad . \quad . \quad . \quad . \quad \text{(II)}$$

where y and t have the same significance as before, while c and z are constants. If two experiments are made with concentrations of serum

y_1 and y_2 and times of appearance of agglutination t_1 and t_2 respectively, then—

$$c = \frac{y_1 y_2 t_1 t_2 (y_1 - y_2)}{y_1^2 t_1 - y_2^2 t_2} \quad . \quad . \quad . \quad . \quad . \quad \text{(III)}$$

and—

$$z = \frac{y_1^2 t_1}{c (y_1 t_1 - c)} = \frac{y_2^2 t_2}{c (y_2 t_2 - c)}. \quad . \quad \text{(IV) and (V)}$$

The curve corresponding to equation (II) is of the type shown in Fig. 2. The action, it will be observed, ceases when a critical concentration is reached (indicated in the figure by the upper asymptote), in this respect recalling that sometimes exhibited by high concentrations of powerful agglutinating sera, which, as is well known, may fail to give rise to agglutination until a dilution of, it may be, 1 in 50 or more is reached.

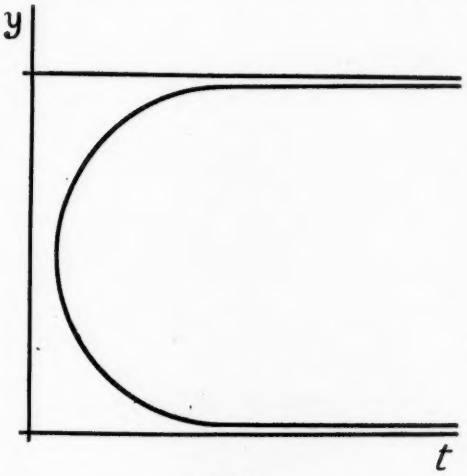

FIG. 2.

The two series of experiments quoted by Arrhenius are given in Table I.

The amounts (in c.cm.) of agglutinating serum employed are indicated by y and the observed agglutination periods (in minutes) by t; in the third column t_1 represents the agglutination periods calculated, according to equation (I), for $yt = 0{\cdot}995$ in the first series and $yt = 0{\cdot}896$ in the second; in the fourth column t_2 gives the same periods calculated, according to equation (II), for $c = 0{\cdot}92$ and $z = 0{\cdot}208$ in the first series and for $c = 0{\cdot}90$ and $z = 0{\cdot}046$ in the second.

TABLE I.

y	t	t_1	t_2	y	t	t_1	t_2
0·080	[20]	12	20	0·008	[140]	112	139
0·035	30	28	32	0·006	150	149	175
0·025	45	40	42	0·0055	165	163	188
0·017	60	58	60	0·005	180	179	204
0·012	90	83	82	0·004	210	224	249
0·008	120	124	120	0·0035	240	256	281
0·005	180	199	189	0·003	300	299	323
0·004	240	249	235	0·0022	420	407	432
0·003	300	332	312	0·002	480	448	472
0·0027	360	369	346				

It is seen that the calculated periods approximate closely to the observed, no matter which of the two equations given above is employed. The experiments do not, however, afford a fair test of the applicability of equation (II), the values of y employed being small compared with cz (= 0·1915 in the first series and = 0·0414 in the second), so that, for these values, the expression to the right of (II) becomes approximately constant and the equation thus resembles (I). With higher concentrations of agglutinin y ceases to be negligible compared with cz, and it becomes possible to determine if the reversal of the curve, shown in Fig. 1, occurs. It is found that experiments exhibiting a wide range of values of y not only fail to give evidence of reversal, but in addition show marked clumping with concentrations of agglutinin exceeding the limiting value at which, according to equation (II), the period of agglutination should become indefinitely prolonged. This is illustrated in the series of observations given below in which *B. dysenteriæ* (Flexner) was agglutinated by rabbit's anti-serum:

TABLE II.

y	t	t_1	t_2
0·5	< 0·20	—	—
0·05	< 0·20	—	—
0·0375	0·25	0·045	0·29
0·0025	0·67	0·68	0·72
0·00075	2·4	2·3	2·3
0·00025	6·5	6·8	6·8

Within the range of concentration of agglutinin covered by the last three experiments both equations are applicable, the calculated periods t_1 and t_2 being found to agree closely with those actually observed (the constants selected were—for equation (I) yt = 0·0017; for equation (II) c = 0·0017, and z = 26). With a concentration of antiserum represented by 0·0375 accurate determination of the period of agglutination became difficult; with still higher concentrations very marked agglutination occurred with a rapidity too great for measurement. But according to equation (II) agglutination ceases when an agglutinin content corresponding to 0·0442 c.cm. of anti-serum contained in 1 c.cm. of fluid is reached: it is obvious, therefore, that this equation fails to represent the relation between concentration of agglutinin and period of agglutination, although in the last three experiments, where y is small compared with cz, the agreement between observed and calculated results is close.

The equation of Arrhenius does not imply any limitation in respect of the occurrence of agglutination when the content of agglutinin is high. Its limits of applicability are chiefly determined, on the one hand, by the diminution in degree and consequent ill-defined character of agglutination with increasing dilution of anti-serum, and, on the other, by the extreme rapidity with which clumping takes place when sufficiently high concentrations of agglutinin are used. Within these limits, which must be ascertained experimentally, equation (I) is valid.

ESTIMATION OF AGGLUTININ.

II. The existence of three variables is implied in equation (I), namely agglutinin (y), time (t) and "effect" (agglutination). The latter is not met with in the equation, but the constant (m) represents a function, not yet determined, of the "effect," and only appears as a constant owing to the present impossibility of estimating quantitatively degrees of "effect." If two of these variables, *e.g.* time and "effect," are measured in fixed units, then the third variable, agglutinin, can be expressed in terms of the first two and is thus obtained in absolute measure. In selecting degree of "effect" only one choice is possible, namely the smallest amount of agglutination which can be recognised. The unit of "effect" is represented by this degree of "effect" occurring in presence of (1) a fixed amount of antigen exhibiting (2) a known degree of agglutinability (both are dealt with further below). If the procedure generally adopted in selecting physical units is followed and the constant in equation (I) is made equal to unity, then the unit of agglutinin is given by the equation—

$$yt = 1 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad \text{(VI)}$$

A serum (undiluted) producing unit "effect" in unit time (1 min.) would thus contain one unit of agglutinin in unit volume (1 c.cm.). If for any other serum $yt = x$, then the normality of this serum is $1/x$. It may be pointed out that the unit of "effect" will not be the same when determined with the naked eye as it is when observed under the microscope, the degree of agglutination required for liminal observation being greater in the former case than in the latter. The ratio of the two units may be determined by making comparative estimations of agglutinin with the same serum. A certain range of variability of result among different observers is to be anticipated, however, since the least amount of agglutination which can be perceived depends upon (1) the acuity of the observer's vision and (2) his power of discrimination, both of which vary in different individuals. Moreover no sharp transition point is met with between an unchanged suspension and one in which agglutination is just recognisable: this applies equally whether agglutination is observed (1) macroscopically at the end of a given period of time, in a graduated series of dilutions of serum, or (2) microscopically, a single dilution being used and the time at which clumping first becomes recognisable noted.

DECAY OF ANTIGEN.

III. The estimation of agglutinin in absolute measure implies, as indicated above, that the antigen employed has an ascertained agglutinability. It is found, however, that both the agglutinin content of a serum and the agglutinability of a bacterial suspension undergo change on keeping. If given amounts of a suspension of bacteria are added at intervals of a few days to samples of a corresponding agglutinating serum (the same quantity of the latter being used in each experiment), it is usually found that the agglutination period increases with lapse of time. If the age (in days) of the serum and suspension, reckoned from the commencement of the experiments, is plotted as ordinate against the period (in minutes) of agglutination as abscissa, a curve similar to

that shown in Fig. 3 is obtained. The lengthening of the time of appearance of agglutination with increasing age of serum and suspension is at first relatively considerable and later becomes relatively slight. As soon as the rate of change has become sufficiently small it is obvious that if now a new serum is prepared, the rate at which this changes on keeping may be determined with a fair degree of accuracy by ascertaining its agglutination period at varying intervals, employing for this purpose the old suspension of bacilli; and conversely, the rate of change of a new suspension may similarly be studied with the aid of the old serum, provided of course that in each case the rate of change to be determined is much greater than that still observable in the course of additional experiments, made with the old serum and the old suspension, designed to explore the further course of the curve given in Fig. 3. The change undergone by an agglutinating serum on keeping will not be considered here; that occurring in a bacillary suspension will alone be dealt with. Some observations upon a freshly prepared suspension of a strain of *B. dysenteriæ* (Flexner) in 0·85 per cent. NaCl solution, containing $1{\cdot}4 \times 10^9$ bacilli per cubic centimetre, were carried out, at 16°C., with the aid of an agglutinating serum, thirteen months old, made from this strain; these are given in Table III and are shown graphically in Fig. 3. Owing to the age of the serum it would appear likely, in view of the type of curve obtained, that the increase of the period of agglutination occurring on keeping is attributable principally to deterioration of the suspension of bacilli. It was found that the observed periods of agglutination at the end of 0, 10 and 26 days were the same, within the limits of experimental error, when the observations were made simultaneously with bacillary suspensions 0, 10 and 26 days old respectively, as when consecutive observations were made upon the same suspension at the end of the same periods of time. In the observations recorded in Table III, therefore, no recognisable fraction of the agglutination period is attributable to deterioration of the serum during the period of experiment; the increase of this period with lapse of time thus appears to be due solely to deterioration of the suspension.

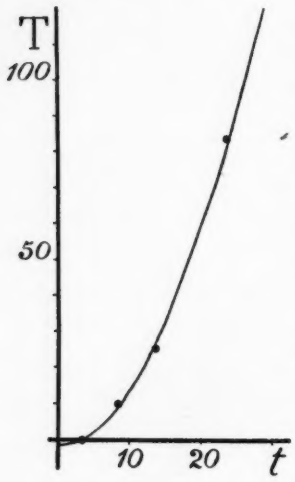

FIG. 3.

TABLE III.

Age of suspension *T*.	Dilution of serum.	Bacilli per 1 c.cm.	Agglutination period *t*.
0 days	1 in 2000	$1{\cdot}4 \times 10^9$	3·4 min.
10 „	„	„	7·5 „
26 „	„	„	14·9 „
83 „	„	„	23·0 „

The experiments of Table III, shown in Fig. 3, seem to lie upon a parabolic curve. The equation for a curve of this type is—

$$T - a = bt^2 \quad . \quad . \quad . \quad . \quad . \quad . \quad \text{(VII)}$$

where T represents age of suspension, time of appearance of agglutination, while a and b are constants. If this relation between T and t is assumed to be valid and the values of a and b are taken at $-1{\cdot}5$ and $0{\cdot}15$ respectively, then the values of t at varying intervals after preparation of the suspension may be calculated. A comparison of observed and calculated values is shown below:

TABLE IV.

T.	$t_{calc.}$	$t_{obs.}$
0 days	3·2 min.	3·4 min.
10 ,,	8·7 ,,	7·5 ,,
26 ,,	13·5 ,,	14·9 ,,
83 ,,	23·7 ,,	23·0 ,,

Since a fair correspondence exists between observed and calculated values equation (VII) may be regarded as valid for the range investigated. The curve exhibits the varying sensitiveness of the suspension at different periods after preparation. The constant a indicates the point upon a theoretical curve at which the examination may be imagined to commence: in other words it represents the equivalent age of the suspension at the actual beginning of experiment. So long as the concentration of agglutinin is unchanged the constant b represents the agglutinability of the suspension, so far as this is exclusively an individual character of the culture used; if, on the other hand, the agglutinability of the suspension remains unaltered, then b is (by equation (I)) proportional to the square of the concentration of serum (agglutinin) employed.

STABLE ANTIGEN.

IV. So long as the agglutination period of a suspension, tested at intervals against an old serum, is observed to be undergoing change, doubt arises as to its stability, and any estimations of agglutinin made with it when the rate of change is small cannot be regarded as possessing more than a comparative value. A fixed agglutination period may be obtained (cp. Dreyer (1906); Gardner (1918)) if the bacteria have been subjected to the action of fixing agents (heat, formaldehyde, thymol, chloroform, phenol). These usually produce at first a diminished sensitiveness, indicated by a lengthening of the agglutination period, sometimes considerable (50 per cent. or more), occasionally absent, after which the period becomes stationary. In some cases the stationary period is again followed by an increase in sensitiveness, indicated by a shortening of the agglutination period. The circumstance that a suspension of bacteria may on keeping exhibit an increase of sensitiveness renders difficult the interpretation of a stationary agglutination period: this may be due either to stability of both suspension and serum, or it may be that a diminution of the agglutinin content of the serum is occurring, but is compensated by an increasing sensitiveness of the suspension. No evidence of a late increase of agglutinability has, however, been observed with formalised suspensions, which are regarded as stable when exhibiting a stationary agglutination period.

AMOUNT OF ANTIGEN EMPLOYED.

V. It has already been stated that in order to define unit of "effect" it is necessary that the antigen employed should be present in fixed amount. In all the experiments given in this article the suspension used contained $1{\cdot}4 \times 10^9$ bacteria per cubic centimetre, observations being made with dark background illumination and a magnification of 350 diameters. Variation of bacterial content, unless extreme, was not found to affect markedly the agglutination period, as is exhibited by the following experiments carried out at 16°C.

TABLE V.

Dilution of serum.	Bacilli per 1 c.cm.	Agglutination period.
1 in 2000	$1{\cdot}4 \times 10^9$	15 min.
,,	,,	14·5 ,,
,,	,,	15·5 ,,
,,	,,	15 ,,
,,	,,	14·5 ,,
,,	$\cdot 14 \times 10^9$	12 ,,
,,	,,	13 ,,
,,	,,	9 ,,
,,	,,	10 ,,
,,	,,	10·5 ,,
,,	,,	10·5 ,,

INFLUENCE OF TEMPERATURE.

VI. Agglutination proceeds more rapidly with rise of temperature, a maximum being reached at 55°C. (Dreyer). In experiments carried out as in Table III, the agglutination periods at 0°C., 16°C. and 38°C. were 60 min., 15 min. and 5 min. respectively. Variation of 1°C. above or below 16°C. affected the agglutination period by $\pm$ 6·2 per cent.

SIGNIFICANCE OF AGGLUTININ DETERMINATION.

VII. In agglutination determinations only that fraction of the total agglutinin content of the serum tested is estimated which corresponds to the particular strain of bacteria used for the suspension. For the estimation of the fraction resulting from immunisation, therefore, a suspension of the particular strain actually used for immunisation can alone be employed; other strains, which may give widely varying results, or even fail to agglutinate, do not constitute a natural standard of reference. Different immune sera can be compared in respect of their agglutinin content only when immunisation has in each case been produced by the same strain. This condition may be fulfilled in experimental work; its fulfilment when dealing with strains isolated from cases of naturally acquired infection cannot be asserted with any degree of certainty, owing to the circumstance that although grouping of strains may be possible, it is nevertheless at present impossible to establish identity of strain.

ABSOLUTE MEASURE.

VIII. Only one observer has, up to the present, attempted to estimate agglutinin in absolute measure, namely Dreyer (1906), who has selected as unit of effect "marked agglutination without sedimentation," observed with the naked eye, and as unit of time 2 hours in the case of *B. typhosus* (*B. dysenteriæ* 4·5 hours), the temperature of experiment being 55° C. A formolised bacterial suspension, exhibiting a stationary agglutination period when tested with a fixed amount of an old agglutinating serum and containing from 3×10^8 to 1×10^9 bacilli per c.cm., is selected as antigen, all other suspensions being standardised in terms of this and a correction co-efficient determined for each. In this method only a single point on the y,t curve, Fig. 1, is investigated, the agglutinin content of the concentration of serum corresponding to this point being, by definition, normal. The reciprocal of this concentration represents the normality of the undiluted serum.

ESTIMATION BASED ON VALUE OF b.

IX. It may happen that the agglutinability of a strain of bacteria will be found on keeping always to diminish in the same manner, according to equation (VII), a suspension containing a fixed number of bacteria per c.cm. when tested against an old serum always giving the same value of b and exhibiting the same curve, of the type shown in Fig. 3. In such a case—and it is to be anticipated that strains will be met with exhibiting this type of specific decay of antigen in the presence, or it may even be in the absence, of fixed conditions of culture and mode of preparation of suspension—it becomes possible to determine in absolute measure the agglutinin content of any old serum, prepared from the strain in question, by determining the value of b for a given dilution of the serum. If that dilution of serum for which $b = 1$ is defined as normal, then the normality of any dilution for which $b = x$ is $\sqrt{x}$, whence the normality of the undiluted serum may be determined. By means of comparative tests with the latter the agglutinin content of a fresh (labile) serum may be ascertained. [The determination of b may also be of value in the identification of strains.] If the strain employed in Table IV were of the type under consideration the normality of the 1 in 2000 dilution of serum would be $\sqrt{0{\cdot}15}\ N = 0{\cdot}387\ N$ and that of the undiluted serum 774 N.

NATURE OF ANTIGEN DECAY.

X. If it is assumed—as is not inherently improbable—that, in a suspension of bacteria, the available antigen, which may be represented by C, is inversely proportional to t, then equation (VII) may be written—

$$T = \frac{B}{C^2} + a \quad . \quad . \quad . \quad . \quad . \quad \text{(VIII)}$$

T and a having the same signification as before, while B is a new constant replacing b. Differentiating we obtain—

$$\frac{dC}{dT} = -kC^3 . \quad . \quad . \quad . \quad . \quad \text{(IX)}$$

where k is a constant $\left(=\frac{1}{2B}\right)$. In this form the decay of antigen appears as a condensation or polymerisation process.

SUMMARY.

(1) For a given "effect" the action between agglutinin and antigen proceeds according to the equation, applicable to a surface action, given by Arrhenius—

$$yt = m,$$

y being concentration of agglutinin, t agglutination period and m a constant.

(2) Using a strain of *B. dysenteriæ* (Flexner), decay of antigen was found to be represented by the equation—

$$T - a = bt^2,$$

T being age of suspension of bacilli, t, as before, agglutination period, a and b constants.

(3) The relation between y and t given in the first equation is applicable to the estimation of agglutinin in absolute measure if the antigen employed is stable; with antigen subject to decay absolute determinations of agglutinin are still possible for a suitable type of constant b, given by the second equation.

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THE PREVENTIVE EFFECT OF INFLUENZA VACCINE.

S. YABE.

Kitasato Institute for Infectious Diseases, Tokyo.

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On the outbreak of the last pandemic of influenza (October, 1918–March, 1919) we first studied the cause of the disease by bacteriological, pathological-anatomical, hæmatological and serological methods, and came to the conclusion that the pandemic was due to Pfeiffer's influenza bacillus, and that the complications were very often caused by various cocci, especially by pneumococci. An account of this work has been published by my colleague, Dr. Okawara, and some co-workers (1918). I continued the study of the immunity to mice, guinea-pigs and rabbits.

Although the toxicity of influenza bacilli is rather strong as compared with the antigenic property, we can render animals immune to a certain degree. Influenza bacilli differ particularly from ordinary pathogenic bacilli in that animals, while rendered moderately immune, are intensely weakened, and even the maximum stage of immunity is not so strong as in the case of other bacilli. This accounts for the fact that a relapse often occurs in human influenza, and that the immune reactions in convalescent serum are slight. From the result of my experiments I could not expect to find that vaccination had as great a preventive effect against influenza as it had against typhoid fever or cholera asiatica. Statistics, however, show that the majority of people who have recovered from influenza are not again attacked by it for a few months, although the amount of immune body in their serum is slight. In view of these facts a slight resistance of a special type might be sufficient to prevent the infection of influenza, and this appears more likely when one considers that there are so many carriers of influenza bacilli during the epidemic, and that real attacks of this disease are occasionally caused by some bodily weakness. I have inoculated three kinds of vaccine, but this report deals only with those cases inoculated with heated vaccine. This was made as follows: 18–20 hours' growth on heated blood agar was emulsified in saline in the proportion of 5·5 mgrm. to 1 c.c. and heated to 60° C. for half-an-hour. When cold this suspension was diluted with one-ninth of its volume of 5 per cent. carbolic saline. When the vaccine was to be bottled it was diluted 9·9 times with carbolic saline, having thus 0·5 mgrm. per c.c. Two inoculations were usually made at an interval of four days subcutaneously in the interscapular region. The first dose for an adult was 0·5 c.c. and the second 1 c.c. These quantities and intervals were determined by experimentation upon myself and others, regard being directed to the bodily reaction and the decrease in leucocytes which facilitates infection.

CONDITIONS NECESSARY FOR JUDGING THE PREVENTIVE EFFECT OF VACCINATION.

To determine the effect of vaccination the conditions under which observation is made should be carefully examined. For statistical purposes the three essential conditions are time, locality and person. In order that full immunity may exist at the outbreak of the epidemic the vaccination should be completed about a fortnight before the outbreak. Owing to the negative phase, as shown by the leucocyte count, a vaccinated person would be more susceptible than a normal if the vaccination was completed just at the beginning of the epidemic, or a few days previously. Vaccination begun during or at the end of an epidemic does not test its preventive effect, since those inoculated would be the residuum of strong people naturally immune.

Regarding locality, care should be taken to select an area which had not been affected at the time of the vaccination.

The personal condition should be strictly observed. The uninoculated control person and the inoculated should live in the same place—for instance in a dormitory. They should be of the same age and sex, with identical occupations, so that their liability to infection may be equally great. For the statistics of a second epidemic, those attacked in the first epidemic should be omitted when considering the preventive effect of the vaccines which were inoculated before the second epidemic.

Thus there are not many good opportunities for determining the preventive effect of vaccination, since most are lacking in one or more of the required conditions. Vaccination with my influenza vaccine was adopted so widely that at the end of the second epidemic (March, 1920) the number of inoculated persons amounted to over half a million. Many variable statistics were reported to me by doctors in the city and in the country. Unfortunately, however, these results were always lacking in some of the conditions necessary for determining the effect of the vaccine. It would be better here to report our own cases in which the three conditions above mentioned were perfectly observed, and to add another series on a different plan from ours, which was examined by my friend, a doctor in the preventive medicine department of a hospital for the families in the Hidachi mining district.

THE RESULT OF VACCINATIONS IN NAGOYA CITY.

In our case, the inoculation was made in two cotton factories in the environs of Nagoya City, one belonging to the Toyoda Company, the other to the Kikuye Company. These two factories are controlled by the same staff. Each have their own workshops, dormitories, laundries and baths, also a power-house, a store, a shop for daily necessaries, a hospital and a school. All these buildings are encircled by a high wall. The clerks, business men, female spinners and weavers, the last two of which constitute over 90 per cent. of all those inoculated, live and work in this enclosure. The female workers are not allowed to go out more than twice a month. As a matter of fact they used to go out once a month. Neither children nor old men live there. In the hospital there are two private doctors with nurses under a matron, entirely at the service of the inmates. We divided these persons into two parties; one was inoculated, the other was left uninoculated as a control.

The personal conditions regarding sex, age, work, mode of life and the prevention of external communication were thus well fulfilled—that is to say, exposure to the danger of infection was about the same in the inoculated party and in the uninoculated control party. As all were living in the same enclosure, having little opportunity for external communication, the local conditions may be approved. As to the condition of time, the intervals between the inoculation and the outbreak of the second epidemic in these two factories and in the surrounding district were as follows:

Days between Inoculation and Epidemic in the Toyoda Factory and in the Surrounding District.

Inoculation.	Date of inoculation.	Epidemic in Toyoda factory.	Epidemic in the surrounding district.
I .	December 25th–26th, 1919 .	Began on January 13th, 1920 .	Began about the end of December, 1919.
II .	December 28th–29th, 1919 .	Ended at the end of January, 1920 .	Ended about the beginning of March, 1920.

Days between Inoculation and Epidemic in the Kikuye Factory and in the Surrounding District.

Inoculation.	Date of inoculation.	Epidemic in Kikuye factory.	Epidemic in the surrounding district.
I .	December 24th–25th, 1919 .	Began on January 8th, 1920 .	Began about the end of December, 1919.
II .	December 29th–30th, 1919 .	Ended on January 28th, 1920 .	Ended about the beginning of March, 1920.

TABLE I.—*The Number of Patients in the Inoculated and Uninoculated Parties in the Factory belonging to the Toyoda Company from December, 1919, to March, 1920.*

	Twice inoculated.	Once inoculated.	Not inoculated.	Total.
Number	2075 .	152 .	237 .	2464
Typical influenza . .	40 (1·93) .	8 (5·26) .	8 (3·38) .	56 (2·27)
Influenza combined with catarrhal pneumonia	3 (0·14) (2 died) .	1 (0·65) .	2 (0·84) (1 died) .	6 (0·24) (3 died)
Per cent. of catarrhal pneumonia to all influenza	(6·97) .	(11·11) .	(20·00) .	(9·68)
Acute bronchitis . . .	57 (2·75) .	10 (6·25) .	13 (5·49) .	80 (3·24)
Pharyngitis . . .	10 (0·48) .	2 (1·32) .	1 (0·37) .	13 (0·52)
Laryngitis	1 (0·05) .	0 .	0 .	1 (0·04)
Tonsilitis	1 (0·05) .	2 (1·32) .	1 (0·34) .	4 (0·16)
Lobular pneumonia . .	0 .	1 (0·65) .	0 .	1 (0·04)
Total number of cases with illnesses of the respiratory tract	111 (5·40) .	24 (15·79) .	25 (10·55) .	161 (6·51)

(The figures in brackets show the percentage of the number of persons in each category of inoculation.)

We observed the cases throughout the second epidemic, and recorded those of influenza and all other illnesses apparently related to this disease which occurred between December 24th–25th, 1919 (the first day of inoculation) and March 10th, 1920. In Table I the persons who were attacked by influenza during the first epidemic are not taken into account.

Chart showing the Number of Illnesses of the Respiratory Tract as above per month, together with the Total Number in the Enclosure from December, 1917, to February, 1920.

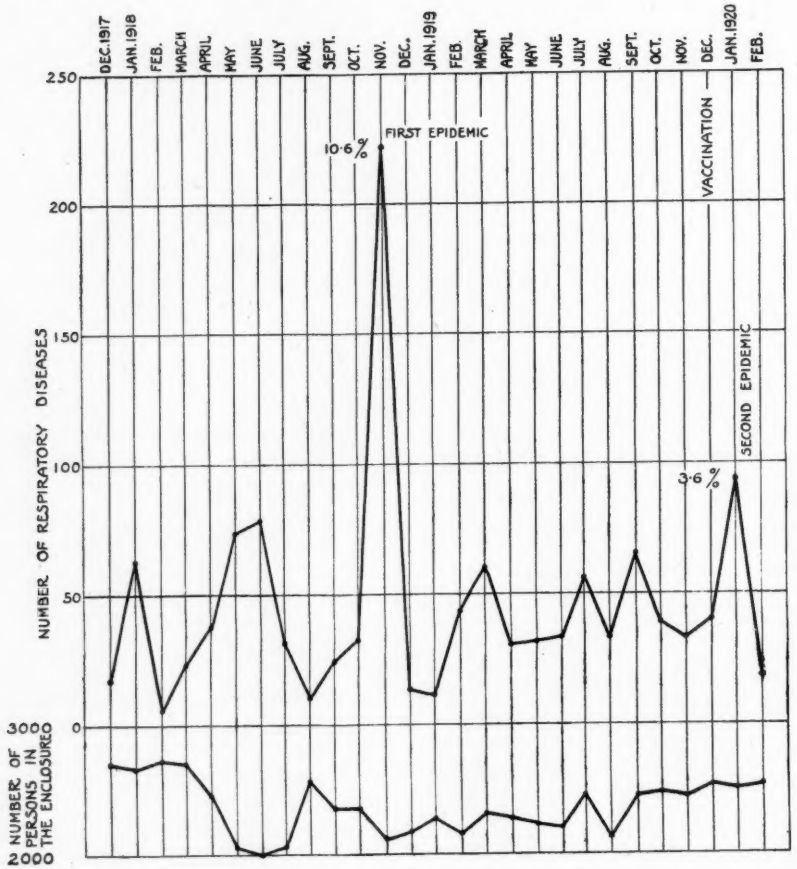

TABLE II.—*The Number of Typical Cases of Influenza in the Inoculated and Uninoculated Parties in the Kikuye Factory during the Second Epidemic.*

	Number.	Typical cases, including catarrhal pneumonia.		Influenzal catarrhal pneumonia.			Deaths.			Average length of fever.
		Number.	Per cent. of total.	Number.	Per cent. of patients.	Per cent. of total.	Number.	Per cent. of patients.	Per cent. of total.	
Twice inoculated	1168	78	(6·68)	17	(21·82)	(1·46)	2	(2·56)	(0·17)	7·27 days.
Once inoculated	447	26	(5·82)	10	(38·46)	(2·24)	1	(3·85)	(0·22)	8·19 ,,
Not inoculated	235	42	(17·87)	16	(38·10)	(6·81)	2	(4·76)	(0·85)	9·05 ,,
Total	1850	146	(7·49)	43	(29·45)	(2·32)	5	(3·42)	(0·27)	8·17 days.

TABLE III.—*Cases slightly affected with Fever, having Acute Pharyngitis, Laryngitis, Tracheo-Bronchitis, which seemed to be Non-typical Influenza. The highest degree of fever in these cases was* 38°C.

	Number.	Non-typical cases.		Average length of fever.
		Number.	Per cent.	
Twice inoculated	1168	11	(0·94)	3·27 days.
Once inoculated	447	10	(2·24)	3·20 ,,
Not inoculated	235	10	(4·26)	3·60 ,,
Total	1850	31	(1·68)	3·35 days.

When the number of patients during the first epidemic is compared with the number of those during the second epidemic the difference is very remarkable. In the neighbourhood outside this factory it is alleged that the second epidemic attacked nearly half the persons who had escaped from the first epidemic. Inside this factory, however, all the above-named illnesses, most of which were influenza, amounted to 10·6 per cent. in the first epidemic (November, 1918), while in the second epidemic (January, 1920), after the preventive inoculation, they reached barely 3·6 per cent. In this percentage 55 remaining persons, who were attacked during the first epidemic, are excluded (*vide* Chart, p. 226).

In the Kikuye Factory there was a fairly large number of cases of influenza having typical symptoms and course. A small number of patients, however, were only slightly ill from some respiratory inflammation. We thus divided them into two groups, one typical, the other atypical (Tables II and III).

In the typical, as in the non-typical influenza, our statistics show that the rate of morbidity of those inoculated is decreased by about a half or a third, though there are some exceptions noted in detail. The most disagreeable complications from catarrhal pneumonia are very remarkably lessened, showing 0·14 per cent. as against 0·84 per cent. in the Toyoda factory and 1·46 per cent. to 6·81 per cent. in the Kikuye factory. I think this is a most interesting point—that the pure influenza vaccine can prevent catarrhal pneumonia and other inflammations of the respiratory tubes which accompanied the epidemic of influenza. But since the so-called catarrhal pneumonia develops only in the course of essential influenza, and since also other inflammations of the respiratory tubes may be due to influenza bacilli, the less frequent appearance of all these sequelæ in persons inoculated with the pure influenza bacilli would be expected. The average length of fever in typical and non-typical cases is always a little shorter among inoculated patients than among those uninoculated.

. To compare the difference in the mortality, there are not, I consider, sufficient cases.

THE RESULT OF VACCINATIONS IN THE HIDACHI MINING DISTRICT.

Next I will quote statistics kindly offered by my friend. In this case the vaccination was begun in the Hidachi mining district just at the beginning of the second epidemic. The relation of the time of the inoculation to the course of the epidemic is as follows: The first case of typical influenza was observed on January 17th, 1920, and the last case on April 15th, 1920. The inoculation was begun three days after the appearance of the first case of influenza and continued on towards the end of the epidemic. Sixty-five per cent. of all the inoculations were made in the first 30 days after the onset of the epidemic, 28 per cent. were made in the next 20 days, the remaining 7 per cent. were inoculated in the last 10 days. Thus the inoculation continued for 60 days, having been begun on the third day after the onset of the epidemic. The epidemic, however, reached its acme between the fortieth and fiftieth day after its outbreak, and lasted to the ninetieth day.

Date.	Cases of influenza in every ten days.	No. of deaths from influenza.	Number of inoculations.		
Jan. 17	1	0			
„ 27	35	0	First inoc.	8484	
Feb. 6	123	1	Second inoc.	7098	
„ 16	185	11			
				15582	(65 per cent. of all inoculations).
„ 26	268	12	First inoc.	4689	
Mar. 8	214	15	Second inoc.	2042	
				6731	(28 per cent. of all inoculations).
„ 18	130	12	First inoc.	1078	
			Second inoc.	549	
				1627	(7 per cent. of all inoculations).
„ 28	58	3			
April 7	30	8			
„ 17	9	1			

The number of inoculated and non-inoculated patients in this mining district was as follows, including the persons who had been attacked in the first epidemic or in the second epidemic before their inoculation :

TABLE IV.—*Number and Percentage of Influenza Cases Complicated by Catarrhal Pneumonia among all the Inoculated and Uninoculated Persons in the Hidachi Mining District.*

	Non-inoculated.	Once inoculated.	Twice inoculated.	Total.
Number of persons	12,501	4,562	9,689	26,752
Number of patients	810	132	116	1,058
Percentage affected	6·4	2·9	1·2	3·9
Number with catarrhal pneumonia complications	130	11	8	149
Percentage of catarrhal pneumonia to patients	(16·1)	(8·3)	(7·0)	(14·2)
Percentage of catarrhal pneumonia to persons	(1·04)	(0·24)	(0·08)	(0·56)
Deaths after complication with catarrhal pneumonia	52	3	1	56
Percentage of deaths to the number of complicated cases	(40·0)	(27·3)	(12·5)	(37·6)
Percentage of deaths to patients	(6·5)	(2·3)	(0·9)	(5·3)

In these figures there are many objections: (1) The persons who had been attacked during the first epidemic were not excluded ; (2) The persons who were attacked just before, or a few days after inoculation were not excluded.

To determine the preventive effect of the vaccine the statistics should be begun at the completion of immunity, excluding persons previously attacked and also those who were attacked during the negative phase after the vaccination.

In order to compare exactly the number of the inoculated patients with those uninoculated, we limited the statistics to certain families attacked by the second epidemic and excluded persons who had been attacked during the first epidemic. The following results were thus obtained. The families here referred to consist of those whose inoculation was completed within 50 days after the outbreak of the second epidemic—that is, by the acme of the epidemic. The patients who were attacked a week after their inoculation are also excluded on account of their negative phase. Thus the local and personal conditions are fairly satisfactory, but the inoculation was too late, being begun only at the outbreak of the epidemic. The condition as to the time for inoculation, therefore, was not satisfactorily fulfilled. Figures in brackets show percentage.

	Non-inoculated.	Once inoculated.	Twice inoculated.
Number of persons	1,845	296	821
Number of influenza cases including catarrhal pneumonia	805	94	60
Percentage of persons	(43·6)	(31·7)	(7·3)
Deaths	55	3	1
Percentage of deaths to patients	(6·8)	(3·2)	(1·4)
Percentage of deaths to persons	(3·0)	(1·0)	(0·1)

In the above tables the morbidity for influenza is one-sixth as great among the twice inoculated persons as among the uninoculated, either in the roughly collected figures or in the more exact figures referring to families. This preventive rate is much greater than in our own test, probably owing to the lateness of inoculation. Thus one must expect a great deal of disagreement if the statistics are collected regardless of the three necessary conditions above proposed. The complications from catarrhal pneumonia are almost half as numerous among the twice inoculated patients as among the uninoculated. But when the rate of complicated cases is calculated on the total number, the decrease is about one-thirteenth among twice vaccinated persons as compared with those unvaccinated. The rates of these complications are similar to those in our own series. The deaths of patients whose cases were complicated with catarrhal pneumonia are very few among those twice vaccinated.

THE REACTION AFTER THE INOCULATION OF THE INFLUENZA VACCINE.

The inoculation was made after persons suffering from diabetes mellitus, nephritis, beri-beri and fever had been excluded, and also those in pregnancy. Almost all the inoculated had some local tenderness on pressure in the inoculated part and a slight feeling of illness for a day in the ordinary course. As a rule cessation from work was not required. In some sensitive people there was, in addition, a general reaction of fever and headache, but even this disappeared in two days.

In the Toyoda Factory 2282 persons were examined after the first inoculation, 5 were found to have ceased work for one day and one had fever over 37·5° C. In the same factory 2130 persons were examined after the second inoculation. Three ceased work for a day and 3 had fever. In the Kikuye factory after the first inoculation 18 out of 1468 persons ceased work but there were no cases of fever. In the Hidachi mining district 12 persons ceased work out of 9762 males and 4489 females after the first inoculation and 16 out of 6538 and 3154 after the second.

Thus the reaction after the inoculation with heated influenza vaccine is so slight that those inoculated need not be off work on account of bodily reaction except in a very few particular cases. As a general rule women have a somewhat stronger reaction than men.

CONCLUSION.

In our statistics, by the use of the heated influenza vaccine, the morbidity of those inoculated was reduced by a half as compared with the rate of those uninoculated. In other statistics, in which the inoculation was begun just at the commencement of the epidemic, and was finished by its acme, the morbidity among the inoculated persons, as compared with that among the uninoculated, was reduced to one-sixth by the use of the vaccine. The complications from catarrhal pneumonia were lessened in the case of inoculated patients to one-half, and deaths to one-seventh. The length of fever was less among the inoculated persons in proportion to the quantity of vaccine inoculated. The reaction after the inoculation was so slight that cessation from work was unnecessary.

Here I express my best thanks to Dr. M. Oshika, in the Toyoda factory, to Dr. M. Takami in the Kikuye factory, and to Dr. I. Kanasawa in the hospital of the Hidachi mining district. The first two kindly managed the inoculation and collected the statistics I wanted, and the last offered me the results of the test according to his plan.

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COMPARATIVE IMMUNITY TESTS WITH SALINE VACCINES AND LIPOVACCINES.

J. PRATT-JOHNSON, M.B., B.S.Lond., D.P.H.Oxon.

From the Bacteriological Department, Lister Institute, London.

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The presence of specific antibodies in the blood of immunised animals is of such general occurrence as to suggest that these substances are important factors in the mechanism of immunity. To what extent this is true is not known. In any case the antibody titre has been employed, perhaps too frequently, as a test of the immunity realised by the vaccination of man and of experimental laboratory animals. The type of immunity so arrived at may bear little or no relation to immunity as defined by ability of the vaccinated animals to withstand the dose or a multiple of the dose of the live organism or organismal protein which would otherwise kill the control unvaccinated animal.

The experiments here detailed were performed primarily for the purpose of studying the relative efficiency of lipovaccines and the ordinary saline vaccine suspensions. For this purpose it was planned to immunise various series of rabbits with vaccines prepared from a member of the Salmonella group possessing marked pathogenicity for the species (Tenbroeck's *Bacillus of Hog Cholera XII*), and, at a fixed interval after the close of immunisation, to test by the lethal dose method the degree of immunity, if any, attained. As we shall see, immunity as defined above was unfortunately not realised with any of the vaccines tried, and the experimental results here recorded bear simply on the lack of association of agglutinin development with genuine resistance to infection by the virulent live organism. In a subsequent paper (1921) some further and more successful attempts to obtain a solid immunity against the virulent strain in question are described.

PREPARATION OF LIPOVACCINES.

A lipovaccine may be defined as a suspension of killed bacteria in an oily vehicle. At first Le Moignic and Pinoy (1916) used mineral oils, which were subsequently replaced by vegetable oils such as olive oil, cotton-seed oil, almond oil, etc. In the French Navy lipovaccines have been employed on a large scale, and their preparation has been investigated by various workers, especially

Whitmore, Fennel and Petersen (1918), Olitsky (1918), and Rosenow and Osterberg (1919). It has been claimed that owing to the diminished toxicity of bacteria suspended in an oily vehicle lipovaccines may be administered in larger doses than saline vaccines, and that by virtue of their slower absorption a more lasting immunity is produced. The chief technical difficulty to be overcome in the preparation of lipovaccine is the removal of the watery envelope around the bacteria to be suspended. To this end various methods have been devised.

Method of Le Moignic.—The watery bacterial emulsion is centrifuged and the supernatant fluid removed. The semi-solid mass of bacteria is then again centrifuged and the remaining drops of watery fluid removed by means of a fine capillary pipette. A hydrophilic fluid of an oily nature, the composition of which is not published, is then allowed to remain in contact with the bacteria for some days, at the end of which time the bacteria, at first white, are penetrated by the oil and are stained reddish by the colouring matter in the oily fluid. The lipovaccine is sterilised by the combined action of "eugenol" and heating to 60° C. for thirty minutes.

Method of Whitmore, Fennel and Petersen.—The bacterial mass, after centrifugalisation, is dried *in vacuo* and finally broken up and incorporated in an oily vehicle by means of constant prolonged shaking in a special machine. The objections to this method are (1) the difficulty of breaking up the dried bacteria finely enough to obtain a uniform suspension, and (2) the question of ensuring the sterility of the vaccine, which is liable to contamination during the drying of the bacterial paste and its transference when dried to the special mixing machine. Further, it is doubtful if this method secures actual penetration of the oil into the bacteria.

Method of Olitsky.—After separation of the watery supernatant fluid by centrifugalisation, almond oil is added and a mixture made mechanically by vigorous shaking with glass beads. This method does not remove the water around the bacteria or secure penetration of the oil. The bacteria remain a greyish-white colour for many months and do not take up the colouring matter present in olive oil. Sterility is secured by heating to 60° C. for thirty minutes, the effectiveness of this low temperature in killing bacteria in an oily fluid demonstrating the presence of water in the lipovaccine.

Method of Rosenow and Osterberg.—The bacteria are collected by centrifugalisation and sterilised by heating or the addition of disinfectants. To the thick watery bacterial paste is then added cotton-seed oil containing 2 per cent. lanoline. The subsequent clearing process is hastened by the presence of the lanoline. The mixture is then heated under reduced pressure at 60° C., the contents being gently shaken until all bubbling has ceased. The water boils away, and the bacteria, which are at first of a greyish-white colour, become yellowish. Heating is continued until the opaque emulsion becomes almost clear.

An objection to this method is the employment of prolonged heating, which, if the brew be a large one, may extend up to three or four hours. The extent to which the antigenic value is affected would require investigation for each organism, and might be expected to vary also with the length of exposure to the heating process.

To ensure sterility of the final lipovaccine it is essential that a sterile bacterial emulsion be used in the first instance. Rosenow and Osterberg's method does secure penetration of the oil into the bacteria.

Eugenol method.—This process was suggested to the writer by a consideration of the analogous process of dehydration and clearing of pathological sections after staining by means of watery solutions. It will be remembered that the presence of mere traces of water produces clouding of the section owing to the incompatibility of water with xylol. Eugenol is a phenoloid substance present to a considerable proportion in commercial clove oil (about 80 per cent.), and has been adopted as an index of the purity of commercial clove oil. It behaves in every way similarly to clove oil but is preferable to it owing to the variable composition of the latter, which is liable to interfere with its clearing action especially when adulterated with alcohol.

The bacterial emulsion is first sterilised by heating to 60°C. for 30 minutes or 53°C. for 70 minutes. It is important to demonstrate its sterility because the subsequent process does not sterilise the thick emulsion. The emulsion is centrifugalised in a sterile tube plugged with cotton-wool and the supernatant fluid pipetted off. The deposit is again centrifugalised until it is packed down hard and the remaining drops of fluid removed with a sterile fine pipette. A few drops of absolute alcohol are then added to the centrifuge tube and the mass rubbed up with a small sterile glass pestle into a thick paste, which is gradually thinned down by adding more alcohol. The whole is again centrifuged and the alcohol removed by a fine pipette. A few drops of eugenol are then added and the sterile pestle again used to form a thick paste which is thinned down by the addition of more eugenol. In 15 to 20 minutes the bacteria are cleared up and penetrated by the eugenol. The lipovaccine is then made up by adding an estimated quantity of the eugenol suspension to sterile olive oil.

While this method has the advantage of simplicity and speedy preparation it is open to the possible objection that the alcohol washings are discarded. The presence of alcohol retards the subsequent clearing process by the eugenol, and also is liable to cause a precipitate when an alcohol-eugenol suspension is added to olive oil owing to the incompatibility of alcohol with olive oil. The antigenic value of the alcoholic washings which are discarded has not been investigated. The employment of alcohol is necessary to ensure the thorough breaking up and dehydration of the bacterial mass.

TECHNIQUE OF STERILITY TEST FOR LIPOVACCINES.

It has been shown that the sterilisation by means of heat of an oily suspension of living bacteria is an entirely different problem to the sterilisation of a watery suspension. The same temperature is required to destroy bacteria in oil as is necessary for bacteria in air. Under these circumstances in the preparation of lipovaccines it is essential that only dead bacteria be used for their preparation, as the high temperature necessary for the sterilisation of oils reduces or destroys the antigenic value of any suspended bacteria.

The addition of an oily fluid to a watery culture medium such as broth does not provide a satisfactory breaking up of the oil even by prolonged

shaking. In view of the importance of definitely ascertaining the sterility of lipovaccines it appeared advisable to work out a technique which would secure a more satisfactory breaking up of the lipovaccine in the culture medium. If equal volumes of 1 per cent. sodium carbonate solution and olive oil be shaken up and then added to a large volume of distilled water a uniform milky fluid is produced. If this fluid be examined under a low power of the microscope it will be seen that the oil is split up into fine globular particles which are suspended in the fluid. If now this picture be compared with a preparation of a few drops of olive oil shaken up in ordinary broth the oil appears as large globular masses, only a small proportion of the oil having been split up into fine globular particles. After numerous experiments the following technique was adopted for testing the sterility of lipovaccines:

0·5 c.c. lipovaccine is pipetted by means of a 5 c.c. pipette into a sterile test-tube containing 2 c.c. of 1 per cent. sodium carbonate solution, and the oil rapidly emulsified by bubbling air through the mixture by means of the pipette. The whole is then sucked up into the pipette and immediately transferred to a 250-c.c. flask containing 50 c.c. of broth and well shaken. The short period of exposure of the oil to the alkali does not appear to be a matter of any practical importance, as *B. typhosus* was found to be still living after an exposure of 45 minutes to 1 per cent. sodium carbonate. It was not found necessary to correct the reaction of the broth to allow for the addition of alkali for the organisms investigated, but this adjustment could be readily worked out to obtain any desired reaction of the final emulsified oil-broth. The preliminary emulsification in this way provides a more efficient sterility test for oily preparations.

COMPARATIVE TESTS OF AGGLUTININ-DEVELOPMENT IN RABBITS IMMUNISED WITH SALINE VACCINES AND LIPOVACCINES.

A 24-hour-growth of the bacillus of Hog Cholera XII was emulsified in saline, shaken, filtered and counted by the dark-ground method. The emulsion was heated at 53° C. for 80 minutes, cooled down and made up to contain 0·5 per cent. carbolic acid. The strength of the final sterile emulsion was 270,000 million per c.c. This stock emulsion was used for the preparation of saline vaccines and lipovaccines.

Saline vaccines.—From this emulsion two saline vaccines were made, viz.:

(*a*) 5000 million per c.c.

(*b*) 10,000 million per c.c.

by diluting with carbolic saline.

Rosenow lipovaccine.—25 c.c. of the above sterile emulsion was centrifuged, the supernatant fluid removed, and sterile beads and 20 c.c. sterile neutralised olive oil containing 2 per cent. lanoline added. The large centrifuge tube was placed in a water-bath at 60° C. and connected up with a water-pump, a reduced pressure of 70-73 mm. of mercury being maintained. During the heating the tube was shaken by hand, the clearing process being completed in 1¼ hours; 55 c.c. of sterile neutralised lanoline olive oil was added and the tube closed with a sterile rubber cork and the whole well shaken. After a satisfactory sterility test a lipovaccine was prepared for use of a strength of 15,000 million per c.c. by the addition of 5 c.c. of the emulsion to 25 c.c. lanoline-olive oil.

Eugenol lipovaccine.—4·4 c.c. of the sterile emulsion was placed in a sterile, plugged centrifuge tube. After centrifuging the supernatant fluid was removed by means of a pipette and the wet deposit again centrifuged, the last drops of fluid being removed by a fine pipette. The deposit was then rubbed up with a few drops of absolute alcohol into a thick paste by means of a sterile glass pestle, the paste being thinned down by the addition of further alcohol. After centrifuging, and pipetting off the alcohol, eugenol was added. At first a few drops were added and the mixture rubbed up into a paste by means of the glass pestle, the volume being subsequently made up to 2·4 c.c. After twenty minutes' treatment in eugenol a lipovaccine of 15,000 million per c.c. was prepared by dilution with sterile olive oil (0·93 c.c. added to 30 c.c. olive oil). The lipovaccine was found to be sterile.

Formalinised suspension.—A suspension was prepared from the original emulsion, and diluted down to a strength of 3000 million organisms per c.c. This suspension was sterilised by the addition of 0·1 per cent. formalin and was used throughout for all the agglutination tests.

Thirty-six rabbits were weighed and sorted out into three groups of similar weight distribution. Before inoculation the serum of all the animals was tested and found to produce no agglutination with the formalinised suspension except in one case where a weak reaction was obtained (1 in 40).

On November 2nd, 1920, the saline vaccine series received 5000 million organisms subcutaneously and the lipovaccine series (Rosenow vaccine 12 animals, eugenol vaccine 12 animals) 15,000 million organisms from their respective vaccines. On November 8th, 1920, the saline series received a further dose of 10,000 millions.

The temperature of all animals was taken night and morning up to November 11th, but except for a rise of 1–4° immediately following the first inoculation no special effects were observed. In no case was any marked local reaction obtained, and no induration or abscess occurred at the site of inoculation.

The serum of each of the thirty-six rabbits was tested on the ninth, thirteenth, sixteenth and twentieth days following the injection of the first dose of vaccine (November 2nd), and on each of these days, also, tests were made of the pooled sera of the twelve rabbits in each group.

The results of the pooled series are appended (Table I).

TABLE I.—*Agglutinin Titres of Rabbits after Inoculation with Saline Vaccines and Lipovaccines.*

Vaccine.	End-point titres on particular days after inoculation.			
	9th.	13th.	16th.	20th.
Saline . . .	1280	2560	2560	2560
"Rosenow" lipovaccine	640	640	320	1280
"Eugenol" lipovaccine	80	160	160	320

I do not propose to detail the figures for the individual rabbits on the days in question, but it may be of interest to note the range of variation experienced in the three series (Table II).

TABLE II.—*Variation in Agglutinin Titre of Rabbits Inoculated with Saline Vaccines and Lipovaccines.*

	No. of animals yielding titres of							
	2560.	1280.	640.	320.	160.	80.	40.	20.
Saline vaccine	9	1	1	1	0	0	0	0
"Rosenow" lipovaccine	3	4	3	1	0	0	1	0
"Eugenol" lipovaccine	0	2	0	1	4	2	1	2

It will be noted that the saline vaccine yielded the best results, while the lipovaccines and especially the eugenol vaccine lagged far behind.

TEST FOR PROTECTION AGAINST THE LIVE ORGANISM.

On the twenty-second day (*i.e.* November 24th) 6 rabbits were taken from each group and given a test dose of 600 living organisms intravenously. The virulence of this organism for the rabbit had been tested previously on numerous occasions, deaths occurring within five to seven days with doses as low as 30 organisms. The actual minimal lethal dose was not ascertained, but from the preliminary experiments made it was clear that the test dose of 600 organisms represented not less than 20 minimal lethal doses. On the day of the test, however, 4 control animals were given 6000, 600, 600 and 60 organisms respectively. All died either on the fifth or on the sixth day.

The annexed table (Table III) shows the results with the vaccinated animals, none of whom survived the test dose. The presence of a high or a low agglutinin-content does not appear to have any influence on the date of death.

TABLE III.—*Date of Death of Infected Rabbits after Vaccination with Saline Vaccines and Lipovaccines.*

Saline vaccine.			Rosenow lipovaccine.			Eugenol lipovaccine.		
Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.
No. 22	5,120	Nov. 28	No. 35	2,560	Nov. 28	No. 46	40	Nov. 30
,, 23	80	,, 29	,, 36	2,560	,, 29	,, 47	20	,, 29
,, 24	2,560	,, 29	,, 37	1,280	,, 29	,, 48	160	,, 29
,, 25	1,280	,, 29	,, 38	320	,, 29	,, 50	80	,, 28
,, 26	640	,, 29	,, 40	320	,, 28	,, 51	160	,, 28
,, 27	10,240	,, 29	,, 41	640	,, 28	,, 52	1,280	,, 29

As it was clear that no protection had been established, it was decided to reinoculate the remaining six animals of each series. On November 30th each received 15,000 million organisms from their respective vaccines, the effect of this second dose being to raise very considerably the agglutinin-titres of the animals receiving the lipovaccine, almost to limits equalling those of the saline vaccine series.

On December 12th a test dose of 210 live organisms was given to each intravenously. At the same time five control animals received respectively 210, 210, 105, 56 and 28 live organisms. All five died between the fourth and

seventh days, and as the annexed table (Table IV) shows, none of the vaccinated animals survived the test dose in spite of the high agglutinin-content of their sera.

TABLE IV.—*Date of Death of Infected Rabbits after Further Vaccination with Saline Vaccines and Lipovaccines.*

Saline vaccine.			Rosenow lipovaccine.			Eugenol lipovaccine.		
Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.
No. 28	640	Dec. 15	No. 39	2,560	Dec. 15	No. 49	320	Dec. 15
,, 29	10,240	,, 16	,, 42	5,120	,, 16	,, 53	5,120	,, 14
,, 30	5,120	,, 15	,, 43	2,560	,, 14	,, 54	2,560	,, 15
,, 31	10,240	,, 15	,, 44	2,560	,, 14	,, 55	2,560	,, 15
,, 32	10,240	,, 15	,, 45	5,120	,, 15	,, 56	320	,, 15
,, 33	2,560	,, 15	,, 34*	—	—	,, 57	640	,, 15

* This animal died in the course of immunisation.

CONCLUSIONS.

(1) Lipovaccines prepared and employed as above stimulate the production of agglutinins to a lesser degree than saline vaccines administered in the same doses.

(2) The presence of a high titre of agglutinins does not necessarily indicate the presence of an established immunity even against small doses of a highly virulent organism.

I here express my acknowledgments to the Governing Body of the Institute for facilities afforded me, and to Dr. Ledingham, Dr. Schütze and others for much helpful advice and criticism.

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FURTHER STUDIES ON THE PRODUCTION OF IMMUNITY IN RABBITS AGAINST AN ORGANISM OF HIGH VIRULENCE FOR THE SPECIES.

J. PRATT-JOHNSON, M.B., B.S.Lond., D.P.H.Oxon.

From the Bacteriological Department, Lister Institute, London.

Received for publication August 6th, 1921.

Previous attempts by the writer (1921) to protect rabbits against a virulent member of the Salmonella group (*B. choleræ suis* XII) by means of killed saline vaccines and lipovaccines having been unsuccessful, the following experiments were carried out with a view to testing the efficacy of immunisation with the live organisms of a related type of low virulence (*B. paratyphosus B*, type "Mutton"). No minimal lethal dose of the virulent organism had been determined, deaths having taken place with regularity after doses containing not more than 30 organisms. Tenbroeck (1918) has put on record certain experiments bearing on this question. He has shown that protection against lethal doses of "Hog cholera XII" can be secured by previous immunisation with live organisms of the "Mutton" but not of the "Schottmüller" type. It appeared therefore of prime importance to ascertain, if possible, the extent of this immunity in parallel experiment with the killed homologous and heterologous vaccines. The following vaccines were prepared:

(1) Killed saline vaccine from *B. choleræ suis* XII.

(2) Killed saline vaccine from *B. paratyphosus B*, type "Mutton."

(3) Living vaccine of *B. paratyphosus B*, type "Mutton."

The living "Mutton" vaccines were prepared and counted on each day of inoculation.

Immunisation.—Six animals were employed for each series. Three injections were given at intervals of seven days, the first injection (March 23rd) subcutaneously, and the second (March 30th) and third (April 6th) intravenously. The doses of the killed vaccines on these three dates were 2000 millions, 2000 millions and 6000 millions respectively, while the three doses of the live "Mutton" vaccines were 30 millions, 30 millions and 300 millions.

Agglutinin-development.—On the thirty-fourth day after the first injection (*i. e.* April 26th) the sera of all the animals were tested against both "Hog cholera XII" and "Mutton" emulsions. Table I shows the agglutination results in end-point dilutions.

TABLE I.

Vaccine group.	Rabbit serial No.	"Hog cholera XII."	"Mutton."
Killed "Hog cholera XII"	81	800	0
	82	100	0
	83	400	0
	84	200	0
	85	400	0
	86	1600	0
Killed "Mutton"	87	0	1600
	88	0	1600
	89	0	800
	90	0	1600
	91	0	1600
	92	0	800
Living "Mutton"	93	50	6400
	94	50	3200
	95	200	6400
	96	50	6400
	97	200	6400
	98	50	6400

It will be noted that only those animals immunised with the live "Mutton" vaccine developed agglutinins for both organisms. The group effect in this case is, however, associated with a remarkably high agglutinin-titre for the homologous organism.

Protection tests.—On April 27th, the day following the agglutination tests above detailed, the inoculated animals received test doses intravenously of the live virulent "Hog cholera XII." Two animals of each series received 200 organisms, two received 1000 organisms, and the remaining two 1,000,000 organisms, while three control animals received respectively 100, 1000 and 1,000,000 organisms. The results are given in Table II.

TABLE II.

Test dose of living "Hog cholera XII."	Killed "hog cholera" vaccine.		Killed "Mutton" vaccine.		Live "Mutton" vaccine.		Controls.	
	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.
200 organisms	81	May 4	87	May 2	93	*Survived*	101	May 2
	82	May 9	88	May 2	94	May 4	—	—
1000 organisms	83	May 3	89	*Survived*	95	*Survived*	102	May 2
	84	May 2	90	May 2	96	May 3	—	—
1,000,000 organisms	85	May 2	91	May 2	97	*Survived*	103	May 1
	86	May 2	92	May 2	98	May 4	—	—

It will be observed that of the six rabbits immunised with the killed "Hog cholera XII" none survived inoculation with the living culture, thus confirming previous results (1921).

Of the six animals immunised with the killed "Mutton" vaccine one survived, while of those which received the living "Mutton" organisms no less than three survived inoculation with living "Hog cholera XII." The series is a small one, and the immunity is not complete in any group, but the result demonstrates unequivocally the possibility of securing solid immunity against a highly virulent organism by vaccination with live members of a related type of low virulence. So far as this experiment teaches, no very obvious relation exists between survival, amount of test dose and date of death. With regard to antibody production, the chief point of interest is a possible association of immunity, not with the high agglutinin-titres following vaccination with the corresponding killed organism, but with the comparatively lowly titres which have developed in the serum as by-products of the action of a live avirulent but otherwise closely related organism.

In all cases the cause of death was verified as due to infection with "Hog cholera XII" by the recovery of this organism in pure culture from the heart-blood.

CONCLUSION.

Experiments are described which demonstrate the possibility of securing the immunity of rabbits against an organism of high virulence by immunisation with a live related organism avirulent for the species. In this respect Tenbroeck's observations are confirmed, and it is further shown that killed vaccines prepared either from the homologous or from a related organism fail to give solid immunity against the live virulent strain.

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THE pH RANGE FOR STAPHYLOCOCCI.

I. WALKER HALL AND A. D. FRASER.

From the Pathological Department of the Bristol University.

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IN the estimations of the relation of hydrogen ion concentrations to the several periods of bacterial life the staphylococcal group has been but rarely employed. It was therefore deemed of interest to examine a series of strains derived from such varying sources as boils, abscesses, sycosis, sinusitis, etc., and from the nose, mouth, skin, genital tract and urine. To the simple determinations of the limiting pH range for these organisms there was added an inquiry as to the association of virulence, the acid or alkali resisting properties, and the preservation of such characters during successive cultivations at pH ranges near the lethal point. It was hoped thereby to discover some means of altering the antigenic capacities of the coccus or its secretions, so as to evolve a more satisfactory means of dealing with the residue of chronic skin and sinus infections which evade the ordinary means of treatment.

METHODS.

A large batch of lait-proto broth (Hall, 1918) was prepared from pure casein, and standardised as to its amino-acid and pH content. The medium was sugar-free, and contained not more than 1 per cent. of NaCl after the final adjustments. This was designed to eliminate the interaction of simultaneous acid and alkaline fermentations, and to provide against the effect of considerable acidity produced during growth. From this standard broth a number of pH ranges were prepared.

Although the changes in pH ranges during growth were determined in several instances, chief attention was devoted to the behaviour of the cocci in an initial hydrogen ion concentration.

The *variations* connected with the use of cultures of differing *ages* was met by the daily transfer of each strain for several days prior to the experiment, and the experimental seeding was made from a four-hour culture in the plain lait-proto broth.

The *amount of inoculum* employed was a standard loopful in all instances, and this was sufficiently large to ensure that the *period of logarithmic increase* fell between four and five hours.

The intensity of growth was determined by the opacity method in this instance, as the period of logarithmic increase formed the subject of another inquiry. The opacity standards were made up in the same broth, with the usual quantities of barium chloride and sulphuric acid. It was found at an early stage that the density of staphylococcal growths is due partly to ether-soluble substances, and that it was necessary to remove them by extraction before the readings were taken. With this precaution the method possesses several advantages, and was found for the specific purpose in view to yield more regular results than bacterial counts. For determination during the maximal periods of growth, however, the use of enumeration methods, though possessing inherent fallacies, was made use of with necessary controls. In this inquiry the readings were taken after twenty-four hours and seven days.

pH INITIAL RANGE FOR STAPHYLOCOCCI DERIVED FROM DIFFERENT SOURCES.

In order to ascertain the pH range for staphylococci and how far it varied for individual strains, a series of broth flasks from the same batch were adjusted with N/HCl or N/NaOH to pH 2·6, 5·0, 7·6, 8 and 10. The seedings were taken from the third subculture of each strain.

Growth after twenty-four hours.—It will be noted from Table I that during the first twenty-four hours certain strains failed to grow in an acidity below pH 5·0, while others derived from a similar type of lesion grew quite easily in pH 2·6. On the other hand, a few of the strains were inhibited by alkalinity beyond pH 8, although the majority grew quite easily at pH 10.

TABLE I.—*The Limiting pH Ranges for Staphylococcal Strains.*

	Source of organism.	Number of strains.	pH concentrations. Twenty-four hours.				Seven days.			
			2·6.	5·0.	8.	10.	2·6.	5·0	8.	10.
S. aureus	Boils	11	8	3	**1**	**10**	10	1	**1**	**10**
,,	Abscess	4	2	2	—	**1**	2	2	—	**1**
,,	Sycosis	1	—	1	—	**1**	1	—	—	**1**
,,	Spinal fluid	1	1	—	—	**1**	1	—	—	**1**
S. albus	Sputum	1	—	1	—	**1**	—	1	—	**1**
,,	Antrum	4	3	1	—	**1**	3	1	—	**1**
,,	Nose	1	1	—	—	**1**	1	—	—	**1**
,,	Skin	1	—	—	—	**1**	1	—	—	**1**
,,	Throat	2	1	1	**1**	**1**	1	1	—	**2**
,,	Urine	2	1	1	—	**2**	2	—	**1**	**1**
,,	Uterus	1	1	—	—	**1**	1	—	—	**1**

Growth after seven days.—When the results of the seven days' incubations are considered, it has to be remembered that Kendall and Farmer (1912, 1913) have shown that in the absence of dextrose, or any other utilisable sugar, the carbohydrate radical of the protein molecule is readily attacked by staphylococci.

Hence in plain broth the same workers found, using neutral red as an indicator, that staphylococci produced acid up to 1 per 100 c.c. for five days, and then alkali to 1 per 100 c.c. for seven days, and 2 per 100 c.c. on the eighth day.

Two strains derived from "boils," one from sycosis and another from urine, all exhibited growth in a higher pH range after seven days, although they failed to do so at an earlier stage. This suggests that our pH 2·6 was simply a delaying and not a lethal range, and that the cocci attained a growth sufficient for registration during the two latter days. We have not burdened the table with the records of the intensity of the growths. A standard opacity was required for the placing of each strain during the readings.

Thus there are variations in the pH limiting range of staphylococci. The differences appear to be inherent in the individual strains, and do not exhibit any relation to the saprophytic or pathogenic characters of the organism. After the first twenty-four hours the cocci commence to grow more easily at the extreme limits.

CHANGES IN SUGAR-FREE MEDIA.

The general action of staphylococci in sugar-free media has already been noted, and the appearance of the secondary fermentation has been observed to coincide with an increase in the amount of growth in the cultures of optimum pH, and an indication of growth in the zones of limiting pH range. It now remains to follow out these changes in the several concentrations and to note how far they are influenced by the respective conditions.

Considering the results as a whole, the extent of alkaline phase is more early and intense the more acid or more alkaline the medium, and less so at the optimal concentration.

For this purpose the organisms were subcultivated several days in the same pH range of broth, and thus finally grown for seven days at 37° C. The changes which follow are, perhaps, more clearly shown when the control and the inoculated media are adjusted to pH 7·5, and the quantities of alkali or acid required are expressed in terms per litre. A single example will suffice to demonstrate the general findings, although the various strains differ also slightly in this characteristic.

TABLE II.—*Alkaline Reactions in Seven Days' Staphylococcal Growths at 37° C. Strain No. 13 from "Boils."*

	Amount of acid or alkali in c.c. per litre required to adjust media to pH 7·5.	
	Medium only.	Medium *plus* staphylococci.
pH 2·6	19·1	14·1
pH 4·0	6·0	4·0
pH 5·0	2·0	1·0
pH **7·5**	**0·2**	**1·0**
pH 9·0	8·0	11·0
pH 10·0	12·0	15·0

ALKALI OR ACID RESISTANT STRAINS.

The variations exhibited by the different strains suggested the possibility of training the organisms to proliferate under higher or lower pH concentrations.

Accordingly a number of strains which grew freely in pH 2·6 broth were subcultured daily for twenty-eight days in pH 2·6 media. Standard loopfuls were transferred on the 1st, 3rd, 7th, 14th, 21st and 26th days into all the pH ranges. Growth could not be obtained in broths more acid than pH 2·6 or more alkaline than pH 10.

A further attempt to produce an experimental adaptation towards the acid or alkaline side was made by allowing the cocci to remain in the pH 2·6 and 10 broths for seven days before transferring them to new broths. It failed equally with the 7th, 14th, 21st and 28th day subcultures. Beyond the limits of pH 2·6 and 10 it seemed impossible to go, so long as the same broth was employed. It was observed, however, that after the 14th day the organisms grew more readily and reached the standard opacity at an earlier hour than hitherto.

CHANGES IN MEDIA CONTAINING ACIDS OTHER THAN HCl.

As it is sometimes necessary to bring media to pH 7·6 by the addition of minute quantities of N/10 HCl, it was considered worth while to test the capacity of the various strains in the presence of other acids. Accordingly a quantity of lait-proto media was prepared and adjusted to 20 amino-acid content, made neutral to phenolpthalein with N/1 NaOH, and then readjusted to the several pH contents with various normal acid solutions. Those selected were nitric, sulphuric, phosphoric, acetic, citric, tartaric, oxalic, lactic, butyric and salicylic. The full details will be given in a subsequent paper upon the action of the diluted acids upon other organisms. The main results with staphylococci indicated a limiting of the pH range by the use of salicylic, sulphuric and tartaric acids. The strains which failed to grow easily in HCl pH 2·6 media failed also in the other solutions. In the pH 5·0 series the salicylic broths were the only set which did not yield good growths. The remainder, in contrast with a retarded proliferation in the 2·6 group, exhibited good opacity within 24 hours. In both groups the nitric acid broths——roughly a dilution of 1 in 50, and 1 in 90 N/10 HNO_3—exhibited a distinct acceleration, resulting in a striking growth at 7 hours and a maximum growth at 24 hours.

Barnes (1921), working with milk as a medium for staphylococcal growth, considers that staphylococci may be divided into sub-groups according to the extent of their enzymic action upon milk proteins, and concludes that there is some relation between the power of liquefying gelatine and that of curdling milk. Similarly we have reason to regard the sub-grouping of these organisms from the standpoint of their acid or alkali-resisting capacities. It may be that our progressing correlation of these features with hæmotoxin production may lend further aid in the recognition of differing types. At all events, there is a sufficiency of data to prompt a closer examination of the antigenic powers of emulsions prepared for therapeutic measures.

SUMMARY.

The general pH range for the staphylococcal group extends from pH 2·6 to pH 10. But individual or group variations occur.

These variations do not exhibit any relation to saprophytic or pathogenic characters.

They do not admit of much experimental adaptation.

The capacity for alkali production in sugar-free media is more evident in lower than higher pH concentrations. By substitution of various acids in the acid series a limiting of the pH range has been produced on the one hand and a definite acceleration of growth on the other, nitric acid being an outstanding adjuvant.

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